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(54) Title: USE OF BASIC AMINO ACIDS TO SOLUBILIZE IMMUNOGLOBULINS

(57) Abstract

A method to solubilize immunoglobulins to practical concentrations in aqueous media employs solubilizing preparations of basic amino acids and/or their salts. For example, histidine, lysine, and arginine and mixtures thereof, in effective concentrations are capable of enhancing the solubility of relatively insoluble proteins and immunoglobulins in general, and in particular, of IgG, especially IgG_3, and of T4.

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USE OF BASIC AMINO ACIDS TO SOLUBILIZE IMMUNOGLOBULINS

Technical Field

The invention relates to the production and purification of proteins and to maintaining their solubility during such production and in storage and formulation. In particular, it concerns use of basic amino acids to solubilize therapeutically and diagnostically important proteins, especially immunoglobulins, and especially those produced in cell culture.

Background Art

Proteins of limited solubility in water present difficult problems during purification, recovery, formulation for therapeutic use, and storage. If sufficient concentrations cannot be obtained, the volumes required for administration of an effective therapeutic dose are above tolerable limits. To obtain efficacy, therapeutic doses of many proteins are administered in the range of several hundred milligrams for a single bolus injection. Therefore low protein concentrations can result in large volumes, causing difficulties in clinical administration to the patient. Preferably administration volumes should be minimized to at least 1-100 ml, preferably less. In order to administer, for example, a 100 mg dose, a solubility of about 2-5 mg/ml is required.

Purification and efficient storage of bulk product or vialed final material may also require that volumes be limited to workable quantities. This too

requires a practical concentration level of at least 2 mg/ml in order to prevent substantial losses due to precipitation when handling reasonable volumes.

This general problem has been recognized, but it is not possible at present to predict under what conditions and for which proteins it will occur, nor is the solution to the problem obvious for a particular protein once the problem is recognized. The present invention resides in the discovery that immunoglobulins, in particular, do not consistently maintain solubility in aqueous media at concentrations greater than 2 mg/ml, that this low level of solubility causes losses in processing, and that the solubility of immunoglobulins can be enhanced by the inclusion in the solution of an effective concentration of basic amino acids.

Use of concomitant solution of amino acids or other biological compounds to stabilize certain specified proteins in aqueous media has been disclosed. U.S. Patent 4,675,183 to Kyowa Hakko claims a method to solubilize interferon using arginine, histidine, lysine, hydroxylysine, ornithine, glutamine, gamma-amino butyric acid, or epsilon-amino caproic acid or their salts. EP Application 217,379, assigned to Mochida and Japanese Application 62/120321, assigned to Eisai, disclose and claim solubilization of tissue plasminogen activator (tPA) using arginine or its salt. U.S. Patent 4,568, 544, assigned to Asahi, claims the use of lysine or arginine to increase tPA solubility. EP Application 242,653, assigned to Eisai, describes tPA solubilization by N-methyl-glucosamine. EP Application 218,112, assigned to Eisai, generally discloses the use of a partial gelatin hydrolysate cross-linked with diisocyanate for tPA solubilization, optionally with the addition of a basic amino acid. Japanese Application 62/153,224, assigned to Green Cross, describes solubilization of plasminogen with sugar alcohols (such as mannitol) or a basic amino acid.

Use of chaotropic materials or covalent conjugation to solubilize membrane-bound proteins has also been described. German Patent Application 3,623,747, discloses the use of cholic acid/glycine or cholic acid/taurine conjugates for this purpose. U.S. Patent 4,511,502 to Genentech describes the solubilizing effect of urea and guanidinium chloride (GnHCl) on recombinantly produced proteins precipitated as refractile bodies. Kurisaki, J. et al, J Biochem (1977) 81:443-449 used GnHCl to solubilize apolipoproteins. Japanese Application 83/38597 describes the use of covalent conjugation of free amino sulfhydryl groups to succinyl glycine to effect solubilization of proteins.

Thus, a review of the art shows that while proteins in general can be solubilized using chaotropic agents or covalent conjugation, processes which result in disruption of the 3-dimensional structure of the protein, solubilization techniques which preserve the structure by employing nondenaturing and conformation conserving solubilization aids are specific for individual proteins, such as plasminogen, interferon and tPA. The present invention provides a workable procedure for immunoglobulins, in particular, and especially for IgG_3.

Disclosure of the Invention

Current techniques in cell culture and protein purification have permitted large-scale production of proteins using mammalian cell culture. In particular, large quantities of monoclonal antibodies can be produced by culturing the hybridomas capable of secreting them in vitro. A variety of culture techniques are available, but in all of them, the antibodies must be purified from the cell culture medium, and, in many cases, formulated into therapeutic compositions. In effecting this purification and formulation, solubility levels which permit practical volumes to be used are required. The invention provides a

method to enhance the solubility of immunoglobulins and related proteins to achieve this result and to increase recovery of final products.

In one aspect, the invention is directed to a method to solubilize immunoglobulins and related proteins in aqueous medium by including an effective amount of one or more basic amino acids or their salts into the mixture. One or more basic amino acids and/or their salts may first be dissolved in aqueous solution and this solution used to dissolve and process the immunoglobulin, or the three components--water, immunoglobulin or related protein and solubilizing amino acid preparation--can be simultaneously mixed. Alternatively, the solubilizing amino acid preparation can be added either in solid or dissolved form to a suspension of the immunoglobulin or related protein.

In another aspect, the invention is directed to compositions of matter which comprise the solubilized immunoglobulin or related protein in the presence of effective amounts of the solubilizing amino acid(s).

Brief Description of the Drawings

Figure 1 shows a flow diagram for purification of IgG_3 from cell culture.

Modes of Carrying Out the Invention

Certain proteins are sufficiently insoluble or are metastable in solution so as to cause difficulties with their purification, formulation, shipment, and storage. Several specific instances of this problem are known, including the two proteins illustrated herein, IgG_3 and the receptor protein T4. However, the invention is not limited to these specific embodiments, but applies to immunoglobulins or other related proteins which encounter solubility problems. In particular, the phrase "immunoglobulins and related proteins" as used herein, in connection with proteins subject to the invention, refers

to proteins which are incapable of stable solubilization in water at levels higher than 2.5 mg/ml. Solubility levels higher than this, which the protein does not normally tolerate, can be obtained using the method of the invention.

Thus the definition of "immunoglobulins and related proteins" for purposes of this application includes any protein, most typically an immunoglobulin, but other proteins as well, which has a solubility in water when a solution of the material is agitated, of less than 2.5 mg/ml.

Immunoglobulins and related proteins can be solubilized, according to the invention method, by inclusion of an effective amount of one or more basic amino acids and/or their salts. The method was developed in connection with the production of monoclonal antibodies by large scale cell culture of their secreting hybridomas; however, it is, of course, applicable to immunoglobulins however prepared. Polyclonal immunoglobulins can be, for example, isolated from serum and the method of the invention is applicable to these polyclonal mixtures. Monoclonal antibodies can be prepared using cell culture methods involving a variety of techniques including perfusion, static maintenance reactor production, shake flasks, roller bottles, and a variety of cell culture techniques known and to be developed in the art. The method of production is not a part of the invention, but provides the problem that is the candidate for solution when high concentrations of immunoglobulins are produced or are desired. Production of IgG_3 presents an especially difficult case.

Similarly, other proteins related to immunoglobulins in their solubility characteristics such as cellular receptors can be solubilized using the method of the invention. Suitable related proteins which are subjects for

the invention method include cellular receptors such as T4.

Similarly, various processes may be used to purify or isolate particular immunoglobulin or related protein fractions. Such techniques include chromatography of various types including gel filtration, ion exchange, affinity chromatography, high performance liquid chromatography, and so forth. In conducting these procedures, tractable volumes must be used, and high solubility levels maintained. Again, the particular methods do not form part of the invention, but rather create the setting in which the method of the invention is useful.

Antibodies of various types are sometimes used therapeutically, for example, for passive immunization, when conjugated to a toxin for therapeutic purposes, or when conjugated to label for localization of tissues. Formulations for administration of immunoglobulin preparations must also maintain high solubility levels of the components in order to restrict the volume of fluid administered to a tolerable level. Related proteins are also used therapeutically and have similar restrictions on solubility levels.

In all of the foregoing cases, solubility levels at a minimum of 3-5 mg/ml are required. Many immunoglobulins and other proteins, such as cellular receptors, do not achieve this solubility level in water solution and require larger volumes than desirable to maintain solubility. In particular, immunoglobulins of the IgG class and, more particularly, those of the IgG_3 subclass, and T4 receptor proteins are difficult to maintain in solution. It appears also that without addition of solubilizing amino acid according to the method of the invention, recoveries of immunoglobulins are diminished, often to a level below that which is economically acceptable. Although no significant increase in

purification yield has been observed with the receptor protein T4, unlike IgG_3, enhanced solubility has been achieved using conditions similar to those for solubilizing immunoglobulins.

The solubility enhancing components of the invention are basic amino acids and/or their salts. "Basic amino acids" are herein defined as those which, when dissolved in water at reasonable concentrations--e.g., 0.1-1M--result in a pH of solution of greater than 7. When these amino acids are used in the present invention, acids can be used to titrate the high pH back to neutrality (i.e., pH 6.5-7.5) if desired. The natively encoded amino acids histidine, arginine, and lysine are preferred, but any basic amino acid, such as, for example, hydroxylysine, ornithine, citrulline, and 2,4-diaminobutyric acid are also included.

The pH of the solution to be used in solubilization can be adjusted, regardless of the form of the amino originally used. Therefore, either the free amino acids or their salts can be employed.

The salts of the amino acids applicable in the invention are the water soluble salts, including the inorganic salts such as the sodium, potassium or ammonium salts or the salts of organic bases such as ethanolamine or triethylamine. Also included are the acid addition salts of the amino functions of the basic amino acid such as the inorganic acid salts, for example, the hydrochlorides or sulfates, or the organic acid salts, such as acetates or citrates. If the solubilization is intended for formulation of therapeutic compositions, of course, pharmaceutically acceptable salts must be used. The solubilizing amino acid preparation must itself be soluble and must be consistent with a pH of the resulting solution in the range of 3-10, preferably 6.5-7.5, if for administration to patients. Additional buffer may be included in the mixture to maintain the pH, if necessary,

however the basic amino acid itself, with proper pH adjustment, behaves as a buffer. These limitations also place restrictions on the nature and extent of neutralization achieved by particular salts of the amino acids in the preparation, as is understood by the ordinary skilled practitioner.

The effective concentration of the solubilizing amino acid preparation depends on the protein to be solubilized and the nature of the solubilizing preparation. In general the degree of solubilization is independent of the basic amino acid concentration once an effective threshold level is achieved. This level can readily be established for a particular combination by a simple optimization experiment. For example, for IgG_3, which has an innate solubility of less than 0.5 mg/ml in water, histidine at a level of 50 mM or greater is effective to permit a solubility of greater than 2.0 mg/ml. To permit flexibility in manipulation, a histidine concentration range of 0.15-0.2 M is preferred. For the T4 receptor, solutions having more than 2.2 mg/ml T4 concentrations are unstable to agitation, but with the addition with 50 mM-150 mM histidine, the solution remains stable to concentrations of T4 up to 17 mg/ml. The solubility levels of T4 in completely unagitated solutions can be up to about 8 mg/ml; however, agitation comparable to that involved in normal handling reduces the level to about 2 mg/ml. Indeed, in one specific instance, shipment of a solution of T4 with a concentration of 2.2 mg/ml was found to contain precipitate upon delivery.

The method to effect solubilization will depend on the situation encountered. If employed to solubilize a formulation, the amino acid preparation may first be dissolved in the aqueous medium and the immunoglobulin or other protein added to the solution either in solid form or dispersed in a small amount of aqueous fluid. Alternatively, solid amino acids or a concentrated solu-

tion thereof can be added to a suspension of the immunoglobulin or other protein, or all three of the components may be simultaneously mixed. The pH may be adjusted by titration at any step. For aiding solubility in purifying and processing immunoglobulins or other proteins produced in cell culture, the amino acid preparation may be added to the harvested medium or the purification buffers directly to maintain solubility in the purification process steps and to improve overall recovery.

In general then, the final solubilized preparation comprises an immunoglobulin or other related protein at a concentration of at least 3 mg/ml and the appropriate effective concentration of amino acid, wherein the pH of the composition is adjusted to the desired value by titration, if necessary.

The following examples are intended to illustrate but not to limit the invention.

Example 1

Effect of Histidine on IgG_3 Solubility

A. IgG_3 was mixed to a suspension of 3-5 mg/ml in various aqueous solutions as shown in Table 1 below, the mixture was agitated, and the presence or absence of precipitation was recorded. As shown, 150 mM histidine was able to prevent the precipitation of the immunoglobulin:

Table 1

Buffer	Precipitation
50 mM phosphate, pH 7	Yes
50 mM phosphate, pH 7 + 150 mM histidine, pH 7	No
50 mM Tris, pH 7	Yes

As shown in the table, even with agitation, the addition of 150 mM histidine prevented precipitation which otherwise occurred.

B. IgG_3 was harvested from cell culture and purified using column chromatography and ultrafiltration at concentrations of >3 mg/ml, using the steps shown in Figure 1. Addition of 150 mM histidine to process buffers was able to prevent otherwise observed precipitation and increase the recovery by 30-40%. A comparison of results with and without inclusion of 150 mM histidine is shown in Table 2. The description of steps refers to that in Figure 1.

Table 2

Recovery of IgG_3 at Each Step of Process

STEP	With Histidine	Without Histidine
Step 1	90%	90%
Step 2	90%	70%
Step 3	85%	50%
Step 4	80%	40%

Example 2

Effect of Histidine on IgG_3 Solubility and Stability

IgG_3 was mixed to a suspension of 2-10 mg/ml in buffer (50 MM Na_2HPO_4 + 200 mM NaCl; pH 7.0) with and without 200 mM histidine.

Solutions were allowed to sit at 4°C for 4-8 hours. Without histidine present the preparation appeared hazy and opaque; when histidine was included, the preparation was clear and colorless.

Fresh samples were prepared and agitated vigorously for 5 minutes. Agitation is a requirement to simulate both shipping conditions and handling during clinical administration. Histidine prevented the precipitation of immunoglobulin; without histidine, a precipitate was evident.

Example 3

Purification of T4 Receptor

A. In one preparation, samples of purified sT4 (dissolved T4) were eluted through an S-Sepharose Fast

Flow column with a resulting concentration of 6.23 mg/ml of sT4 in a buffer solution of 0.5 M NaCl and 50 mM sodium phosphate, pH 7. 150 mM histidine was added to one sample and samples both with and without added histidine were agitated on a Fisher Vortex Genie 2. The sample without histidine visibly precipitated and remained cloudy for over 24 hours. The sample with histidine remained in solution and clear for at least 24 hours.

B. In an additional, similar preparation, 150 mM histidine was added following a normal purification production run, resulting in a concentration of 17.1 mg/ml. The preparation could be stirred without evidence of precipitation and no precipitation resulted after cross-country commercial shipping.

Claims

1. A method to solubilize an immunoglobulin or related protein in aqueous medium, which method comprises

admixing with a dispersion of the immunoglobulin or related protein in said medium, an amount of a solubilizing amino acid preparation effective to solubilize said immunoglobulin or related protein,

wherein said preparation consists essentially of one or more basic amino acids and/or their salts.

2. The method of claim 1 wherein the immunoglobulin is of the class IgG and the related protein is a cellular receptor.

3. The method of claim 1 wherein the basic amino acids are selected from the group consisting of histidine, arginine, lysine, hydroxylysine, ornithine and citrulline.

4. The method of claim 6 wherein the basic amino acid is histidine.

5. A method to solubilize an immunoglobulin or related protein in aqueous solution, which method comprises

adding said immunoglobulin or related protein to an aqueous solution containing a solubilizing amino acid preparation at a concentration effective to permit the solubilization of the immunoglobulin or related protein,

wherein said amino acid preparation consists essentially of one or more basic amino acids and/or their salts.

6. The method of claim 5 wherein the immunoglobulin is of the class IgG and the related protein is a cellular receptor.

7. The method of claim 5 wherein the basic amino acids are selected from the group consisting of histidine, arginine, lysine, hydroxylysine, ornithine and citrulline.

8. The method of claim 7 wherein the basic amino acid is histidine.

9. A composition of matter which comprises an aqueous solution of immunoglobulin or related protein at a concentration of more than 3 mg/ml and of a solubilizing amino acid preparation

wherein said amino acid preparation consists essentially of one or more basic amino acids and/or salts thereof, said preparation in a concentration effective to solubilize the immunoglobulin or related protein.

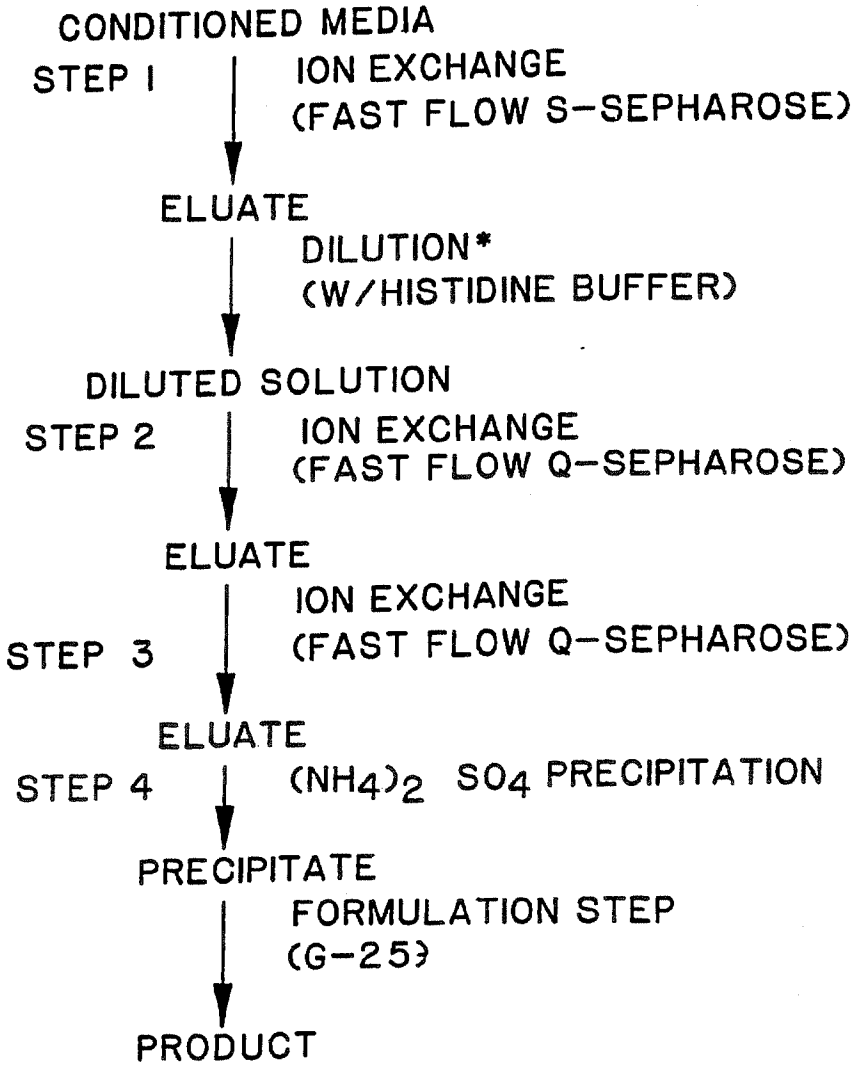
10. The composition of claim 9 wherein the immunoglobulin is of the class IgG and the related protein is a cellular receptor.

11. The composition of claim 10 wherein the cellular receptor is T4.

12. The composition of claim 9 wherein the basic amino acids are selected from the group consisting of histidine, arginine, lysine, hydroxylysine, ornithine and citrulline.

FIG. 1

PURIFICATION OF IgG_3 FROM CELL CULTURE



*PRECIPITATION PROBLEMS BEGIN AT THIS POINT IN PROCESS AND WOULD REMAIN THROUGHT IF HISTIDINE WERE NOT PRESENT.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/05516

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/02, 39/395, 47/00
U.S. Cl.: 530/350, 387; 424/85.8

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	530/350, 387; 424/85.8

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Databases: Chemical Abstracts Services Online (File CA, 1967-1989); File Biosis, 1969-1989). Automated Patent System (USPAT), 1975-1989.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
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X Y	US, A, 4,597,966 (ZOLTON) 01 July 1986, "Histidine Stabilized Immunoglobulin and Method of preparation."	1-10,12 11
Y	US, A, 4,568,544 (HASEGAWA) 04 February 1986, "Aqueous Solution of a Tissue Plasminogen Activator Dissolved Therein at an Increased Concentration and a Method."	1-12
Y	Archives of Biochemistry and Biophysics, 1976, volume 173, Sophianopoulos, "Solubility of Hemoglobins by Sedimentation , Equilibrium, and Antisickling Compounds, pp. 517-527	1-12
Y	Journal Biochemistry, volume 81, 1977, Kurisaki, "Selective Solubilization of Apolipoproteins from Hen's Egg Yolk Very Low Density Lipoprotein With Guanidine Hydrochloride and Urea," pp. 443-449	1-12

* Special categories of cited documents: [10]

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07 MARCH 1990	30 MAR 1990

International Searching Authority	Signature of Authorized Officer
ISA/US	Jon D. BERNSTEIN Ngoc Ha Nguyen Ha Nguyen

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
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Y	US, A, 4,675,183, 23 June 1987. KATO, "Method for Solubilization of Interferon."	1-12
Y	Science, volume 209, 25 July 1980 Terhorst, "Biochemical Analysis of Human T Lymphocyte Differentiation Antigens T4 and T5," pp. 520-521.	1-12